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Beyond bystanders: Myeloid cells in chronic lymphocytic leukemia

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ABSTRACT

Tumor-promoting inflammation and escape from immune-mediated tumor destruction have been recognized as hallmarks of cancer, and myeloid cells are key players in these processes. By exploiting the tremendous plasticity of myeloid cells, tumors induce a variety of tumor-supportive and immunosuppressive cell phenotypes like tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs). The relevance of these cell types in hematopoietic malignancies has only recently gained a stronger attention. Chronic lymphocytic leukemia (CLL) is a malignancy of mature B cells that expand in secondary lymphoid organs and the bone marrow, and accumulate in the blood of patients. A large body of evidence suggests that the interactions between CLL cells and non-malignant cells in the tumor microenvironment play a key role in the pathology of this disease. CLL is associated with an inflammatory milieu and defective immune responses. A severe skewing of myeloid and T cells toward leukemia-supportive and immunosuppressive phenotypes have been observed in patient samples and the Eμ-TCL1 mouse model of CLL. Myeloid cells were thereby shown to enhance survival of CLL cells and contribute to apoptosis-resistance, to suppress anti-tumoral immunity, and to be involved in immune deficiency of leukemia patients. In addition, treatment regimens that are currently used for CLL target not only directly the malignant cells, but have also an impact on non-malignant bystander cells, including myeloid cells. This review summarizes current literature on these aspects and gives a perspective on how our current knowledge might be used to design novel immunotherapeutic approaches that can be combined with CLL-targeting drugs to achieve better therapeutic responses in CLL patients.

1. Introduction

1.1. Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is one of the most common adult leukemias in the western world that affects approximately 3.9 in 100,000 people. It is a disease of the elderly with a median age at diagnosis of about 72 years and higher incidence in males compared to females. (Zenz et al., 2010) CLL results from an accumulation of mature $CD5^+$ B cells in peripheral blood (PB), bone marrow (BM), and secondary lymphoid organs. The majority of patients is asymptomatic at the time of diagnosis, but upon examination, lymph node (LN) enlargement is detected in many of them. (Nabhan and Rosen, 2014) CLL diagnosis is defined by lymphocytosis with more than 5 million lymphocytes/mL in PB. (Hallek et al., 2008) Lower degrees of lymphocytosis are diagnosed as monoclonal B-cell lymphocytosis, an asymptomatic state that shares many features with CLL but has no organ involvement and is thought to be a CLL precursor state. (Rawstron et al., 2008) CLL cells are characterized by a special immunophenotype that differentiates them from other B-cell malignancies, being positive for the T-cell antigen CD5 and for B-cell surface antigens CD19 and CD23, while showing low expression of CD20. (Hallek et al., 2008; Ginaldi et al., 1998) The marker profile of CLL cells, including CD25, CD69 and CD71, matches that of antigen-experienced B cells with expression of some memory B-cell markers like CD27. (Damle et al., 2002) However, the cellular origin of CLL is still under debate. (Chiorazzi and Ferrarini, 2011; Seifert et al., 2012; Oakes et al., 2016)

Among many pathways that are aberrantly regulated in CLL cells, B-cell receptor (BCR) signaling plays a central role in CLL pathogenesis. (Burger and Chiorazzi, 2013) BCR stimulation has been shown to enhance cell survival and upregulate anti-apoptotic proteins, such as myeloid cell leukemia 1 (MCL-1), in CLL cells. (Petlickovski et al., 2005) Moreover, CLL is characterized by chronically activated BCR signaling and gene expression profiling of leukemic cells in lymphoid tissues showed an activated BCR signature in these cells. (Herishanu et al., 2011a) This is also evident by the higher degree of phosphorylation of the protein kinases LYN, SYK, and extracellular signal-regulated kinase (ERK) in CLL compared to normal B cells. (Burger and

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Chiorazzi, 2013) Mutations affecting BCR elements are very rare in CLL, (Quesada et al., 2012) suggesting that the aberrant BCR signaling in CLL is rather induced by chronic antigen stimulation in the microenvironment. This is supported by the fact that more than 30% of CLL cases display stereotyped BCR sequences, signifying that common antigens or autoantigens might drive CLL cell selection and expansion. (Messmer et al., 2004; Stamatopoulos et al., 2007; Agathangelidis et al., 2012) In addition, BCR signaling in CLL can be induced autonomously in an antigen-independent manner. (Duhren-von Minden et al., 2012; Iacovelli et al., 2015)

1.2. Current and emerging therapies in CLL

In contrast to other leukemias, therapeutic intervention in CLL may not be required until the patient progresses to symptomatic disease. (Gribben and O'Brien, 2011) This is based on clinical trials showing no benefit of early versus late intervention in indolent CLL cases. (Dighiero et al., 1998) Historically, alkylating agents, such as cyclophosphamide and bendamustine, in addition to purine analogues like fludarabine comprised the backbone therapy for CLL patients. (Zenz et al., 2010) Complementing these chemotherapeutics with anti-CD20 antibodies (e.g. Rituximab), which was collectively termed chemoimmunotherapy, resulted in improved overall and progression-free survival. (Hallek et al., 2010; Fischer et al., 2012) Therefore, chemoimmunotherapy is currently considered the standard first line therapy in CLL. (Hallek et al., 2010) Besides, allogeneic stem cell transplantation is considered the only curative treatment for CLL patients. (Dreger, 2009) As previously mentioned, research in the last 10 years shed light on the importance of BCR signaling as a central pathway in CLL pathogenesis. Several small molecule inhibitors targeting BCR downstream kinases have been shown to impact on CLL cell survival in vitro and in vivo. (de Rooij et al., 2012; Hoellenriegel et al., 2011; McCaig et al., 2011) The Bruton's tyrosine kinase (BTK) inhibitor Ibrutinib and the phosphatidylinositol 3 kinase δ (PI3Kδ) inhibitor Idelalisib showed very promising results in relapsed CLL patients. In addition to their favorable safety profiles, these inhibitors are of special benefit for poor outcome groups harboring TP53 mutations or 17p deletions. (Byrd et al., 2014; Furman et al., 2014; Burger et al., 2014) Of interest, these highly potent BCR inhibitors result in pronounced shrinkage of LNs paralleled with significant lymphocytosis in treated patients which is most likely due to mobilization of CLL cells from lymphoid organs to PB. (Woyach et al., 2014; Brown et al., 2014) Furthermore, selective B-cell lymphoma 2 (BCL-2) inhibitors represent another promising class of small molecule inhibitors for CLL. (Souers et al., 2013) In addition, cellular therapies involving chimeric antigen receptor (CAR)-modified T cells targeting CD19 are currently under investigation in CLL. (Porter et al., 2011)

1.3. Microenvironment in CLL

The longstanding view was that CLL arises from the accumulation of non-proliferating apoptosis-resistant B cells in PB and lymphoid organs. However, seminal work by Messmer et al. using deuterated water labeling demonstrated that CLL cells exhibit a proliferation rate of 0.1–1.75% per day and higher proliferation rates correlated with worse prognosis. (Messmer et al., 2005) In LNs and BM, CLL cells form structures called pseudofollicles or proliferation centers which involve considerable proliferation of CLL cells. (Granziero et al., 2001; Ratech et al., 1988; Schmid and Isaacson, 1994; Soma et al., 2006) In these structures, CLL cells are thought to encounter antigens and receive survival and proliferation signals from their cellular niche. (Caligaris-Cappio and Ghia, 2008) Moreover, comparative transcriptional analysis of CLL cells isolated from LNs, BM and PB clearly showed that interplay between leukemic cells and bystander non-malignant cells in lymphoid tissues is the major driver for apoptosis- and chemoresistance and tumor expansion mediated by nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling. (Herishanu et al., 2011a) Therefore, CLL has been regarded as a two-compartment disease in which cells in the proliferation centers act as the source for the small, quiescent lymphocytes that are long-lived and therefore accumulate in PB. (Caligaris-Cappio and Ghia, 2008) In addition, Calissano et al. identified intraclonal heterogeneity of proliferation rates between CLL cell subsets harboring different activation markers or chemokine receptors. (Calissano et al., 2009) Accordingly, they proposed a dynamic model in which CLL cells that receive activating signals in the tissue site are released to blood stream. Lack of survival signals in PB stimulates upregulation of chemokine receptors, such as chemokine C-X-C motif receptor 4 (CXCR4), which mediate migration of those cells back to tissue sites. (Calissano et al., 2011) Importantly, interference with microenvironmental interactions in lymphoid organs leads to mobilization of CLL cells to the periphery. (Ponader et al., 2012a; Chen et al., 2016) This phenomenon has been of major relevance for assessing the success of multiple targeted therapies in CLL, which will be discussed in detail in an upcoming section. (Woyach et al., 2014; Brown et al., 2014; Cheson et al., 2012)

The CLL microenvironment comprises a complex network of multiple cell types that play distinct roles in supporting CLL cell survival and evasion of immune attack. In addition to mesenchymal cells, fibroblasts, T- and natural killer cells, innate myeloid cells represent key players in the CLL microenvironment. (Burger, 2011)

In the blood of CLL patients, abnormal distributions and phenotypes of myeloid cells have been observed, and their numbers were shown to be of prognostic relevance. (Gustafson et al., 2012; Maffei et al., 2013; Jitschin et al., 2014) In addition to multiple in vitro observations, (Burger, 2011) the strongest evidence for a pathogenic role of myeloid cells in CLL comes from mouse models, where the depletion of monocytes and macrophages by liposomal clodronate, or of granulocytes by Ly6G-specific antibodies prevented or slowed down disease development. (Hanna et al., 2016; Galletti et al., 2016; Gätjen et al., 2016) Accordingly, the importance of myeloid cells in CLL is based on three main aspects. They contribute to (i) apoptosis-resistance and enhanced survival of malignant cells, (ii) evasion of anti-tumoral immunity, and (iii) immune deficiency in leukemic patients, as summarized in Fig. 1. Therefore, myeloid cells are becoming increasingly attractive as therapeutic targets in CLL, similar as in other tumor entities. Furthermore, myeloid cells most likely contribute to the clinical effects of multiple CLL-targeted therapies. The current review will discuss the organization of the myeloid lineage, its role in CLL pathophysiology, and potential use as target for CLL therapy.

2. The myeloid cell lineage and its organization

Myeloid cells are a complex network of immune cells that play a key role in protection from pathogens, elimination of dying cells and mediating tissue remodeling. (Richards et al., 2013) Therefore, they are crucial for proper function of innate and adaptive immunity. Myeloid cells develop from multipotent hematopoietic stem cells that progressively differentiate into more specialized progenitor cell types which are restricted to either the granulocytic lineage or the monocytic lineage, including monocytes, macrophages and dendritic cell subsets. (Fogg et al., 2006; Onai et al., 2007; Naik et al., 2007; Geissmann et al., 2010; Hashimoto et al., 2011; Hettinger et al., 2013) The major myeloid cell types and their function under inflammatory conditions will be briefly described below.

2.1. Neutrophils

Neutrophils are the most prevalent of the polymorphonuclear (PMN) granulocytes and of human leukocytes in general. The BM releases 1–2 × 10^{11} neutrophils to the blood each day and retains almost 20-fold of that number as a post-mitotic reservoir. (Borregaard, 2010; Dancey et al., 1976) To accommodate such a high production demand, two thirds of the myeloid compartment in the BM is dedicated to

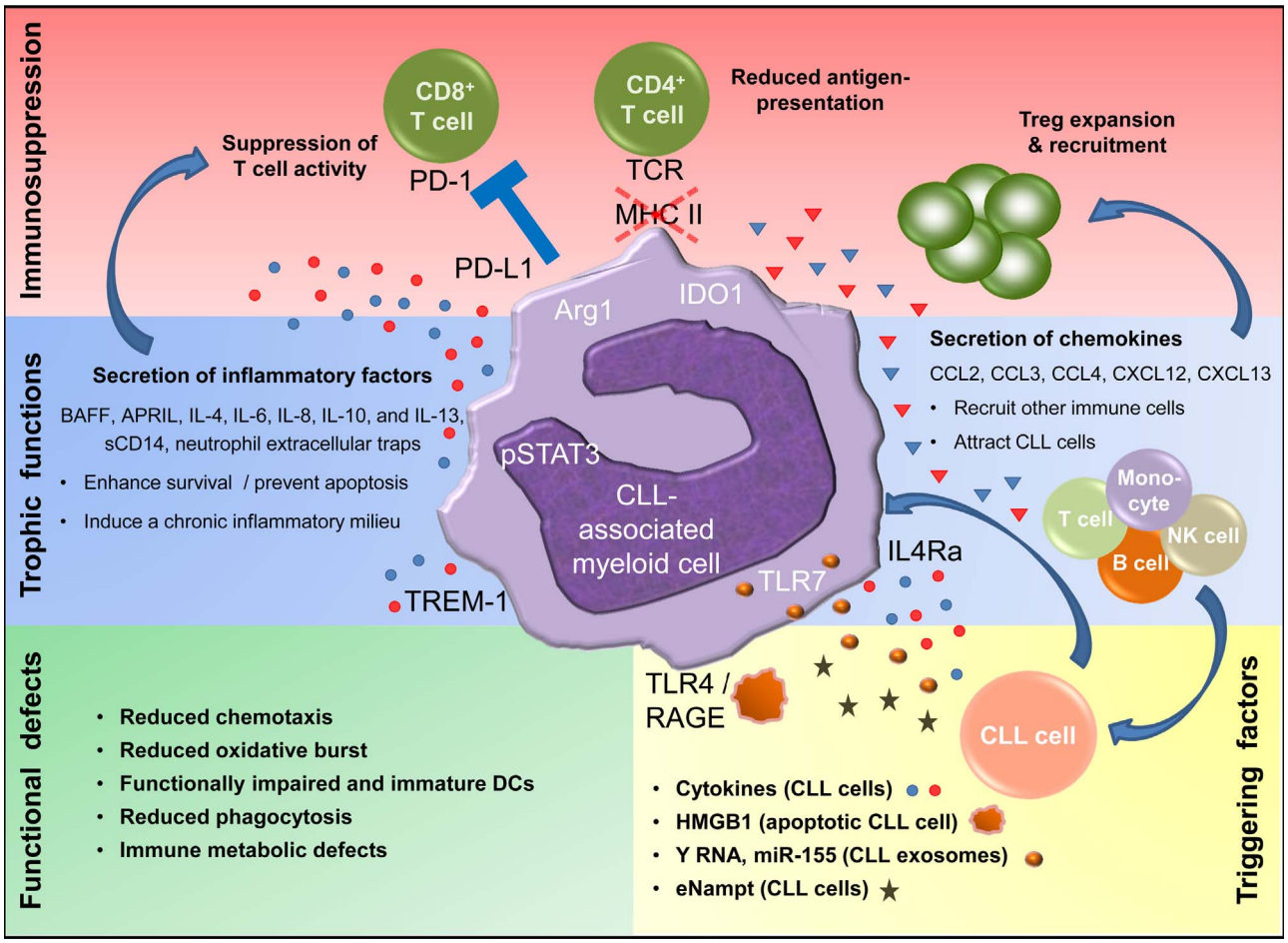

Fig. 1. Contributions of myeloid cells to CLL pathobiology. Myeloid cells nourish and support CLL cells via secretion of inflammatory factors and chemokines (blue box). Secreted factors, immunomodulatory ligands and enzymes expressed by myeloid cells impair adaptive immune responses, which leads to escape from anti-tumoral immunity (red box). CLL-associated myeloid cells are functionally defective contributing to immunodeficiency in patients (green box). These features of myeloid cells are induced by CLL cells via several identified factors and pathways (yellow box).

neutrophil production. (Borregaard, 2010)

Blood neutrophils are able to translocate to tissues; in fact this can happen in a time as short as a few seconds. (Mauer et al., 1960) Once in tissues, and classically in contexts of acute inflammation, neutrophils are efficient at killing their target microorganisms. (Segal, 2005) The pathogen is phagocytosed and neutrophils release anti-bacterial proteins and reactive oxygen species (ROS) into the phagosome. Alternatively, extracellular killing is achieved by releasing the contents of neutrophil-intrinsic granules to the extracellular space. In addition, neutrophils can be induced to de novo synthesize and release a wide array of pro- and anti-inflammatory cytokines, such as tumor-necrosis factor α (TNFα) and transforming growth factor β (TGFβ).(Cassatella, 1999; Scapini et al., 2008) Consistent with the ability to interact with and instruct other cell types, neutrophils are now recognized as a node in orchestrating both innate and adaptive immunity. (Mantovani et al., 2011)

2.2. Monocytes

Monocytes originate from macrophage/dendritic cell progenitor cells in the BM and subsequently migrate to peripheral circulation. They are broadly divided into two distinct subsets, inflammatory and patrolling monocytes, which differ in expression of chemokine receptors and other surface molecules. (Shi and Pamer, 2011) Inflammatory monocytes express high levels of the chemokine C−C motif receptor 2 (CCR2) and migrate to sites of inflammation in response to its chemokine C−C motif ligand 2 (CCL2), where they differentiate into macrophages or dendritic cells. Patrolling monocytes play a key role in monitoring vascular endothelial integrity and contribute to tissue regeneration after inflammation. (Gordon and Taylor, 2005; Carlin et al., 2013) While human monocyte subsets are defined based on differential expression of the two cell surface molecules cluster of differentiation (CD) 14 and CD16, murine monocytes are classified as $Ly6C^{high}CD43^{low}$ inflammatory and $Ly6C^{low}CD43^{high}$ patrolling monocytes. (Ziegler-Heitbrock et al., 2010) The two subsets show differences in additional phenotypical markers, in their transcription profiles and half-lives (0.2 days for inflammatory monocytes compared to 2.2 days for patrolling monocytes). (Yona et al., 2013; Ingersoll et al., 2010; Wong et al., 2011) Furthermore, the developmental relationship between inflammatory and patrolling monocytes was recently uncovered by fate mapping studies which showed that murine inflammatory monocytes are the obligatory upstream precursors of the patrolling subset. (Yona et al., 2013)

2.3. Macrophages

Macrophages are terminally differentiated myeloid cells that are widely distributed in different body organs where they have distinct functions. Under physiologic conditions, they play a key role in maintaining tissue homeostasis by engulfing apoptotic bodies, and producing growth and angiogenic factors, such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF).(Gordon and Taylor, 2005) In addition, they play a crucial role under inflammatory states starting with pathogen clearance and then

contributing to tissue repair and regeneration at later states. (Soehnlein and Lindbom, 2010) Historically, macrophages were thought to originate only from blood monocytes that differentiate upon entering tissue sites. (van Furth and Cohn, 1968) However, it has recently been reported that most tissue-resident macrophages under homeostatic conditions are derived from yolk sac progenitors that seed into organs at early embryonic stages and are maintained locally with minimal contribution from blood monocytes. (Yona et al., 2013; Epelman et al., 2014; Ginhoux et al., 2010; Hashimoto et al., 2013) On the other hand, monocyte-derived macrophages are mostly generated in response to inflammation. (Shi and Pamer, 2011)

Macrophages exhibit profound plasticity that enables them to differentiate into various phenotypes depending on the signals in their local microenvironment. (Gordon and Taylor, 2005) In response to immunogenic signals (e.g. bacterial lipopolysaccharides) or inflammatory cytokines (e.g. interferon gamma; IFNγ), macrophages are polarized towards stimulatory phenotypes that are essential for pathogen defense. This includes release of ROS, high expression of inflammatory cytokines (e.g. interleukin (IL)-12) and upregulation of major histocompatibility complex class II (MHC-II).(Gabrilovich et al., 2012; Biswas and Mantovani, 2010) Following this inflammatory stage, macrophages switch to immunomodulatory phenotypes and contribute to wound healing and restoring tissue homeostasis. (Murray and Wynn, 2011) These two macrophage phenotypes have been classically termed M1 (classically activated) or M2 (non-classically or alternatively activated) and can be recapitulated in vitro by stimulation with lipopolysaccharides and IFNγ, or IL-4, respectively. (Gordon and Martinez, 2010; Stein et al., 1992) The differentiation of macrophages is tightly controlled by distinct signaling pathways; e.g. signal transducer and activator of transcription 1 (STAT1) controls M1 polarization while STAT3 and STAT6 control M2 polarization. (Sica and Mantovani, 2012) These two polarization states also differ in their chemokine profiles; while M1 macrophages produce T helper (Th) 1 cell–attracting chemokines, such as chemokine C-X-C motif ligand 9 (CXCL9) and CXCL10, M2 macrophages express the Th2 chemokines CCL17, CCL22 and CCL24. (Mantovani, 2008; Martinez et al., 2006) Yet, it should be noted that this binary classification of macrophages into M1/M2 phenotypes can oversimplify the complexity of macrophage response to a broad range of stimuli under physiological and pathological conditions. (Murray et al., 2014)

2.4. Dendritic cells

Dendritic cells (DCs) represent another major type of terminally differentiated myeloid cells that originate from common DC precursor cells and have been recently shown to be independent of monocytes and other myeloid lineages. (Liu et al., 2009; Schraml and Reis e Sousa, 2015) Yet, monocytes can differentiate into DCs under inflammatory conditions. (Randolph et al., 1999; Auffray et al., 2009) One subset of DCs termed plasmacytoid DCs (pDCs) are morphologically similar to plasma cells and secrete high amounts of IFNα in response to virus exposure. (Colonna et al., 2004) All other DCs are referred to as classical DCs which survey tissues in search for foreign or damage-associated antigens, which they process and present to T lymphocytes. (Villadangos and Schnorrer, 2007) Therefore, DCs play key roles in bridging innate and adaptive immunity and due to their superior antigen presentation ability they are considered the most professional antigen-presenting cells. DCs also play a seemingly-opposing role in inducing immune tolerance, for example by inducing and maintaining regulatory T cells (Tregs).(Darrasse-Jeze et al., 2009) The tolerogenic role of DCs is linked to their maturation status. In contrast to mature DCs that express high levels of MHC class II and co-stimulatory molecules, immature DCs harbor low levels of these proteins and rather overexpress immunosuppressive factors, such as indoleamine 2,3-dioxygenase (IDO1), which translates into their tolerogenic nature. (Maldonado and von Andrian, 2010; Finkelman et al., 1996; Steinman and Nussenzweig, 2002)

3. Cancer-associated myeloid cells

The main myeloid cell populations in the tumor microenvironment are tumor-associated macrophages (TAMs), monocytes, neutrophils (sometimes referred to as tumor-associated neutrophils, TANs), and DCs. Monocytes and neutrophils in cancer patients often have an immature phenotype and express immunosuppressive genes and are therefore named monocytic or granulocytic (also polymorphonuclear) myeloid-derived suppressor cells (MDSC), respectively.

Due to their highly plastic nature, myeloid cells in tumors can act as a double-edged sword. (Allavena et al., 2008) On one hand, myeloid cells are able to recognize tumor-associated antigens and to initiate inflammatory processes, including the recruitment and activation of other immune cells that can contribute to anti-tumor immune responses. If properly activated, macrophages may control initial tumor development, for example by enhancing antibody-mediated killing of tumor cells. (Mantovani et al., 1977) There are several reports on anti-tumoral activities of neutrophils as well. In patients with early stages of small-cell lung cancer, a hybrid monocyte-neutrophil cell subset was described that was able to stimulate both antigen-specific and non-specific T-cell responses and to secrete TNFα and IL-12. (Singhal et al., 2016) An upregulated expression of the hepatocyte growth factor (HGF) receptor MET in neutrophils in response to tumor-derived TNFα lead to neutrophil localization to the tumor site in the lung and killing of tumor cells using neutrophil-derived ROS. (Finisguerra et al., 2015)

Despite their anti-tumoral potential, the largest body of current literature suggests that myeloid cells in most cancers have tumor promoting functions. (Gabrilovich et al., 2012) These tumor-supportive features of myeloid cells can be broadly classified into tumor-trophic functions that directly enhance tumor cell survival, proliferation, invasion and metastasis, or immunosuppressive functions that foster tumor growth by sabotaging anti-tumoral immune functions. The contribution of myeloid cells to these aspects in tumors, and particularly in CLL, will be discussed below.

4. Myeloid cells harbor tumor trophic functions

4.1. Tumor-associated macrophages

In most cancers, TAMs are the most abundant tumor infiltrating immune cells. As discussed above, they have a highly plastic nature with varied activities. (Allavena et al., 2008) Whereas M1 inflammatory macrophages have tumoricidal activity, TAMs mostly differentiate into an M2-like tumor-supportive phenotype, (Biswas and Mantovani, 2010) which has been shown to facilitate tumorigenesis via different mechanisms, such as induction of angiogenesis and promotion of metastasis. (Qian and Pollard, 2010) This can happen in response to soluble factors secreted by immunoregulatory cells in the tumor microenvironment or by the tumor cells themselves. For example, IL-4 secreted by T helper cells in mammary carcinoma polarizes macrophages towards tumor-promoting phenotypes. (DeNardo et al., 2009) IL-10, IL-4, and IL-13 secreted by Tregs induce M2 polarization of human macrophages. (Tiemessen et al., 2007) Recent studies showed that aberrant tumor metabolism results in increased lactic acid production which induces TAM generation in a hypoxia-inducible factor 1α (HIF1α)-dependent manner. (Colegio et al., 2014) However, it should be noted that TAMs exhibit marked heterogeneity in their phenotypes in different tumors. In addition, TAMs present at different locations in the microenvironment have been reported to harbor distinct phenotypes and functional states. (Movahedi et al., 2010) The diversity of TAMs is thought to be due to a differential education by the tumor to generate specific niches for optimal support of cancer cells. (Lewis and Pollard, 2006)

4.2. From monocytes to nurse-like cells – TAMs in CLL

The first indication of myeloid cell contribution in CLL pathology was by Burger et al. who demonstrated that blood monocytes differentiate in vitro under the influence of CLL cells into nurse-like cells (NLCs) that protect malignant cells from spontaneous apoptosis. (Burger et al., 2000) This model has been extensively used for drug testing and simulating the tissue microenvironment in CLL. (Ponader et al., 2012a; Buchner et al., 2010; Schulz et al., 2013) NLCs have been shown to attract CLL cells by the release of chemokines, such as CXCL12 and CXCL13. (Burger et al., 2000; Burkle et al., 2007) They further secrete proteins, such as B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), which enhance CLL cell survival. (Nishio et al., 2005) Moreover, NLCs stimulate CLL cells to release chemokines, such as CCL3 and CCL4, which are important for the recruitment of other leukocytes. (Burger et al., 2009) IL-4, −6, −8, −10, and −13, which were also implicated in viability and proliferation of CLL cells, are secreted by NLCs and other cell types in the microenvironment of CLL. (Francia di Celle et al., 1996; Moreno et al., 2001; Plander et al., 2009; Chaouchi et al., 1996)

There are several lines of evidence showing that CLL cells actively shape their microenvironment. Healthy donor monocytes when cocultured with CLL cells differentiate to NLCs to provide survival support for the malignant cells ex vivo. (Tsukada et al., 2002a) Previous work from our group demonstrated that CLL cells actively induce an inflammatory milieu by enhancing the secretion of CCL2 and other cytokines from monocytes which further results in the recruitment and activation of myeloid cells and other immune cells. (Schulz et al., 2011) In addition, we showed that CLL cells induced shedding of CD14 by monocytes, and soluble CD14 enhanced survival of CLL cells in vitro, although the underlying mechanism of this is so far not understood. (Seiffert et al., 2010) Elevated serum levels of many of the above mentioned cytokines, as well as blood monocyte counts were shown to correlate with clinical outcome of CLL patients and suggest a pathological role for these cells and their secreted factors. (Gustafson et al., 2012; Fayad et al., 2001; Yan et al., 2011; Herishanu et al., 2011b) Among the cytokines that were found to be upregulated in CLL is macrophage migration inhibitory factor (MIF) which is responsible for recruitment of macrophages to the sites of disease development. Interestingly, targeted deletion of MIF delays development of CLL in the Eμ-TCL1 mouse model and results in prolonged survival of mice. (Bichi et al., 2002; Reinart et al., 2013)

4.3. Mechanisms of nurse-like cell induction

Although the role of NLCs in supporting CLL cell survival is well documented, the mechanism of their generation remains largely elusive. Jia et al. have recently shown that the release of high-mobility-group-protein B1 (HMGB1) from apoptotic CLL cells may be responsible for NLC formation through its interaction with Toll-like receptor (TLR) 4 and the receptor for advanced glycation end products, RAGE. (Jia et al., 2014) There is further evidence that the extracellular form of nicotinamide phosphoribosyltransferase (eNAMPT), a molecule that exhibits cytokine-/adipokine-like properties, is important for differentiation of resting monocytes, polarizing them toward tumor-supporting M2 macrophages that secrete many cytokines with pro-survival activities, such as IL-6 and IL-8. Both intracellular and extracellular NAMPT levels are increased in cells and plasma of CLL patients and eNAMPT production by CLL cells is enhanced upon B-cell receptor, TLR, and NF-κB signaling pathway activation. (Audrito et al., 2015) We have further shown that CLL-derived exosomes and their contained non-coding Y RNA, upon uptake by monocytes and macrophages induce the secretion of inflammatory cytokines, such as CCL2, CCL3 and CCL4. (Haderk et al., 2017) This response was mediated by TLR7 and pharmacological inhibition of TLR signaling in Eμ-TCL1 mice slowed down leukemia development and might therefore be of therapeutic interest.

4.4. In vivo counterparts of NLCs

NLC differentiation of monocytes is an in vitro phenomenon, and the in vivo equivalents of these cells and their developmental relationship to other myeloid cells are still unclear. Based on surface marker expression and similarity in gene expression profiles, NLCs were suggested to resemble tumor-associated and M2-type macrophages. (Ysebaert and Fournie, 2017; Filip et al., 2013) In accordance with that, $CD68^{high}$ $CD163^{high}$ myeloid cells are found in the spleens and LNs of CLL patients in close contact to leukemic cells. (Tsukada et al., 2002b; Boissard et al., 2016)

While these studies shed some light on the contribution of myeloid cells in CLL, one main limitation is that they were mostly based on cells isolated from blood which have been shown to differ in many aspects from cells residing in LNs or BM where the complex interactions with immune cells take place. (Herishanu et al., 2011a; Audrito et al., 2015) To overcome these limitations, we used the Eμ-TCL1 mouse model of CLL and clearly demonstrated that monocytes accumulate in all organs that are affected by disease. (Hanna et al., 2016) We further showed that their recruitment depends on CCR2, the receptor of CCL2. And the main monocyte type that was found at the tumor sites were $Ly6C^{low}$ patrolling monocytes. (Hanna et al., 2016) Gene expression profiling of these cells showed increased expression of triggering receptor expressed on myeloid cells 1 (TREM-1) and many inflammatory cytokines, suggesting that monocytes impact on CLL development in this model by inducing an inflammatory milieu. Elevated TREM-1 signaling as well as serum cytokine levels were further observed in CLL patients, confirming the relevance of inflammation in this disease. (Schulz et al., 2011) Concomitant with the above observations, depletion of monocytes and macrophages using the lysozyme M–directed myeloid-specific DTR (LysM-Cre/iDTR) model or clodronate liposomes delayed CLL development. (Hanna et al., 2016; Gätjen et al., 2016)

4.5. Tumor-associated neutrophils

There is increasing evidence that suggests a pathological involvement of granulocytes in many different tumor entities. As a disease, cancer was shown to impact on several stages in the life of a neutrophil, starting from enhancement of granulopoiesis and neutrophil mobilization from the BM, extending to increasing the lifespan of neutrophils, and culminating in affecting the polarization of neutrophils towards tumor-associated phenotypes (reviewed by Coffelt et al., 2016) Similar to the binary classification of macrophages into M1 and M2 phenotypes, some studies report neutrophils to polarize towards either an anti-tumor N1 phenotype, or a pro-tumor phenotype, referred to as N2 neutrophils. (Fridlender et al., 2009; Piccard et al., 2012) The latter have been shown to support tumors by enhancing angiogenesis, increasing proliferation, and decreasing cancer cell senescence. (Yang et al., 2004; Di Mitri et al., 2014)

The involvement of granulocytic cells in CLL pathology has not been extensively studied so far. Very recently, both Hirz et al. and Podaza et al. showed that neutrophils protect CLL cells against drug-induced and spontaneous apoptosis in vitro. (Hirz and Dumontet, 2016; Podaza et al., 2017) As underlying mechanism, neutrophil extracellular traps (NETs) were suggested as mediators of CLL, but not normal B-cell viability. (Podaza et al., 2017) In another study using the Eμ-TCL1 mouse model, Gätjen et al. performed gene expression profiling of unsorted tumor stroma from the spleen of leukemic Eμ-TCL1 mice and healthy control mice. (Gätjen et al., 2016) They observed an enriched granulocyte gene signature in the tumor stroma, and concomitantly reported increased numbers of granulocytes in the spleens of leukemic mice. Interestingly, when they depleted granulocytes for four weeks using an anti-Ly6G antibody in Eμ-TCL1 mice at early CLL stage, they observed a decrease in tumor progression, which shows that granulocytes provide important tumor support. As they further observed that granulocytes from the Eμ-TCL1 mice express higher levels of the B-cell

stimulating factors BAFF and APRIL compared to wild-type mice, they suggested that neutrophils might be supporting CLL cell viability by these secreted factors.

5. Myeloid cells harbor immunosuppressive activities

5.1. Tumor-associated macrophages and nurse-like cells

Myeloid cells are essential for presenting tumor-derived antigens to T cells and keeping T cells in an activated state. However, in the tumor microenvironment, they rather acquire a phenotype to suppress T-cell activity against the tumor. (Gabrilovich et al., 2012) One mechanism of how TAMs do this is via the production of high amounts of arginine- and tryptophan-catabolyzing enzymes, like arginase 1 (ARG1) and IDO1, respectively. ARG1 and IDO1 activities deplete the local microenvironment of L-arginine and tryptophan which are essential for proper function of cytotoxic T cells. (Rodriguez et al., 2004; Cannon et al., 2015) Furthermore, IDO1 activity causes expansion of immunosuppressive Tregs. (Fallarino et al., 2006) TAMs also produce insufficient amounts of immunostimulatory cytokines, such as IL-12, resulting in sabotaging cytotoxic T-cell responses by tumors. (Sica and Mantovani, 2012) On the other side, IL-10 produced by TAMs promotes Th2 responses and enhances the activity of Tregs. (Gabrilovich et al., 2012) Tregs in turn produce IL-10 which acts in a positive feedback loop resulting in further promotion of immunosuppressive activities of TAMs. (Quatromoni and Eruslanov, 2012)

Expression of IDO1 has been observed in NLCs from CLL cocultures, as well as $CD68^{high}$ $CD163^{high}$ myeloid cells in CLL LNs. (Giannoni et al., 2014) In cocultures, NLCs have been further shown to suppress T-cell proliferation, which was at least partially rescued by an IDO1 inhibitor. (Giannoni et al., 2014) Whether the $CD68^{high}$ $CD163^{high}$ myeloid cells in LNs or spleen of CLL patients are functionally immunosuppressive still requires further investigation.

5.2. Myeloid-derived suppressor cells

MDSCs are a heterogeneous population of immature myeloid cells that accumulate in BM, spleen, LNs, PB and tumor sites of cancer patients and tumor-bearing mice. They differ from normal immature myeloid cells in healthy individuals by their highly potent immunosuppressive activity. (Ostrand-Rosenberg and Sinha, 2009) Murine MDSCs are phenotypically defined by the co-expression of CD11b and Gr1 (Ly6C and Ly6G) and can be generally classified into monocytic MDSCs (Mo-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs). Mo-MDSCs are classically defined as $CD11b^{+}Gr1^{int}Ly6C^{high}Ly6G^{-}$ cells which resemble phenotypically normal monocytes, while PMN-MDSCs are more similar to granulocytes and are defined as $CD11b^{+}Gr1^{high}Ly6C^{low}Ly6G^{+}$ cells. (Gabrilovich et al., 2012) Mo-MDSCs comprise precursors of monocytes and DCs. (Narita et al., 2009; Corzo et al., 2010) However, the limited marker panel available to define MDSCs makes it difficult to identify the developmental stage at which they were arrested.

One of the main causes of MDSC accumulation in tumors is the increased inflammation at tumor sites. (Ostrand-Rosenberg and Sinha, 2009) Inflammatory mediators produced by tumor cells, such as IL-1β, IL-6 and prostaglandin E2, are capable of inducing and attracting MDSCs in addition to potentiating their suppressive activity. (Song et al., 2005; Bunt et al., 2007; Zhang et al., 2009) MDSCs can sabotage anti-tumoral immunity via different mechanisms. Similar to the mechanisms described above for TAMs, they have an enhanced production of ARG1 and IDO1 which disrupts T-cell function. (Gabrilovich et al., 2012; Yu et al., 2013) In addition, MDSCs overexpress inducible nitric oxide synthase (iNOS) which can inhibit T-cell function by increasing production of nitric oxide resulting in reduced JAK3/STAT5 signaling in T cells and induction of their apoptosis. (Bingisser et al., 1998; Rivoltini et al., 2002) Hypoxia in tumors can also induce the upregulation of inhibitory ligands, such as programmed cell death 1 ligand 1 (PD-L1), in MDSCs which inhibit T-cell proliferation and anti-tumor activity by interaction with the immune checkpoint molecule programmed cell death protein 1 (PD-1) on T cells. (Noman et al., 2014) MDSCs can in addition regulate other myeloid cells in tumors for example by secreting IL-10 which polarizes macrophages into M2 phenotypes. (Ostrand-Rosenberg et al., 2012)

There is little known about the role of MDSCs in CLL. Accumulation of $CD14^{+}HLA\text{-}DR^{low}$ monocytic MDSCs is reported in CLL patients' blood and it correlates with tumor progression and poor prognosis. (Gustafson et al., 2012; Jitschin et al., 2014; Liu et al., 2015) These MDSCs express high levels of IDO1, which contributes to their suppression of cytotoxic T cells in vitro and induction of Tregs. (Jitschin et al., 2014) CLL-derived exosomes have been shown to induce an MDSC phenotype in monocytes, characterized by HLA-DR down-regulation and upregulation of immunosuppressive proteins. (Haderk et al., 2017; Bruns et al., 2017) Bruns et al. showed that exosomal transfer of miR-155 modulates several miR-155 target genes like FOXO3 in monocytes, which contributes to CLL-mediated MDSC induction. (Bruns et al., 2017) They further showed that MDSC induction can be controlled by vitamin D that negatively regulates miR-155 expression in CLL cells. (Bruns et al., 2017) Our recent work demonstrated that CLL-derived exosomes induce a suppressive phenotype in monocytes and macrophages with high levels of IDO1 and PD-L1 expression. (Haderk et al., 2017) We further showed that this was mediated by exosomal Y RNA which binds as ligand to endosomal TLR7 after uptake of CLL-derived exosomes by monocytes or macrophages.

5.3. Dendritic cells

In addition to monocytes and macrophages, DCs contribute to immunosuppression. As professional antigen-presenting cells, they are capable of detecting tumor antigens and presenting them to T cells initiating tumor-specific responses. However, tumors develop multiple means to evade DC-mediated immune reactions. Among them, inhibition of DC maturation and accumulation of tolerogenic immature DCs is a commonly used strategy of different tumors. (Perrot et al., 2007) In CLL patients, circulating DCs are reported to have an immature phenotype, being severely defective and unable to stimulate effective T-cell responses, highlighting their importance in immunosuppression. (Orsini et al., 2003) By exploiting the antigen-presenting capacity of DCs, many groups have focused on the development of DC-based vaccines as immunotherapeutic approaches for CLL, which is not covered here, but already reviewed by Palma and colleagues. (Palma et al., 2008)

5.4. Immunosuppression by myeloid cells in the Eμ-TCL1 mouse model

Most of the above described data originated from PB samples of CLL patients or cocultures of primary leukemic cells. For studying the more relevant tumor sites for CLL, the secondary lymphoid tissues, mouse models are essential. Studies in the Eμ-TCL1 mouse line have provided a great amount of information about the microenvironmental regulation of CLL in vivo. In these mice, macrophages and monocytes infiltrate the peritoneal cavity where the leukemia cells initially accumulate. These infiltrating cells show a higher expression of the M2 markers CD206 (mannose receptor), CD124 (IL-4Rα) and ARG1, and higher levels of STAT3 phosphorylation in response to IL-10 stimulation, as well as lower expression of the costimulatory receptor CD86. (Hanna et al., 2016) This is in line with gene expression data from Gätjen et al. showing that tumor-exposed stroma, harbors a significantly enriched M2 macrophage signature. (Gätjen et al., 2016) At later stages of disease, CLL progresses mainly in the spleen of the Eμ-TCL1 mice, resulting in splenomegaly. Interestingly, monocytes, mainly patrolling monocytes, and to lower extent DCs accumulate in the spleen with CLL progression. (Hanna et al., 2016) These monocytes and DCs show

aberrantly high expression of the inhibitory molecule PD-L1. (Hanna et al., 2016) Furthermore, blocking specifically PD-L1 in vivo also restores $CD8^+$ T-cell cytotoxicity and normalizes T-cell cytokines and proliferation, allowing control of CLL development. (McClanahan et al., 2015)

6. Immune defects of tumor-associated myeloid cells

As CLL cells impact on myeloid cells to alter their phenotype and to perform different functions in favor of tumor growth, disease progression in CLL patients is associated with declined myeloid cell immune function. As early as in the 1970s, scientists observed decreased activity of neutrophils from CLL patients, such as decreased myeloperoxidase activity. (Zeya et al., 1979) CLL is accompanied by general immunosuppression and susceptibility to infections. Itälä et al. compared characteristics of neutrophils of CLL patients who experienced infections, to patients without infections. (Itala et al., 1996) Interestingly, they observed decreased chemotaxis and oxidative burst in neutrophils of infected CLL patients. It would be very interesting to know if the infected individuals had more immunosuppressive and immature neutrophils, as their neutrophils were not functioning properly. Others also showed that neutrophils of CLL patients have impaired bactericidal but not fungicidal activity in vitro. (Kontoyiannis et al., 2013) These observations point toward CLL neutrophils losing typical neutrophil functions, while probably acquiring tumor-supportive capabilities.

Plasmacytoid DCs support the activity of effector immune cells and are therefore critical for anti-viral immunity and anti-tumor responses. In CLL patient samples and the Eμ-TCL1 mouse model, a reduced number and functional impairment of pDCs was observed that correlated with disease progression. (Saulep-Easton et al., 2014) This study further showed that TNFα or TGFβ inhibition restored pDC numbers and therefore offers a new strategy to improve immune competence in CLL.

There are several reports that describe defective properties of monocytes and macrophages, including their ability of phagocytosis and antigen presentation, as well as alterations in the secretion of cytokines which is necessary for the recruitment of immune effector cells. Qorraj et al. recently suggested that immune metabolic defects in CLL monocytes might impact on their function. (Qorraj et al., 2017) They showed that a reduced glycolytic competence of CLL monocytes which is mediated by BTK and the immune checkpoint protein PD-1, impacts on their phagocytic ability. Inhibition of BTK worsened this phenotype whereas immune checkpoint blockade with anti-PD-1 antibodies improved phagocytosis rates of CLL monocytes. Gene expression profiling of human and murine CLL-associated macrophages and monocytes further suggested an immature phenotype with decreased immune competence. (Hanna et al., 2016; Bhattacharya et al., 2011) In leukemic Eμ-TCL1 mice, a loss of MHC class II-expressing macrophages and DCs was observed, suggesting impaired antigen presentation in CLL. (Hanna et al., 2016) These mice further show a severe skewing of $CD8^+$ effector T cells, with an enrichment of cells showing features of exhaustion, including the upregulation of immunoregulatory proteins, like PD-1, reduced effector functions, and impaired ability to form immunological synapses. (McClanahan et al., 2015; Ramsay et al., 2008; Hofbauer et al., 2011) A potential involvement of myeloid cells in inducing T-cell exhaustion in these mice was further demonstrated by Hanna et al. (Hanna et al., 2016)

7. Therapeutic approaches to target myeloid cells

7.1. Impact of BTK and PI3K blockade on myeloid cells

The development of novel targeted therapies has improved the clinical outcome of CLL patients considerably. Interestingly, one of the most successful new drugs, the BTK inhibitor Ibrutinib, induces the mobilization of CLL cells from LNs to PB, where they are deprived from microenvironmental support. (de Rooij et al., 2012; Ponader et al., 2012b) There is increasing evidence that Ibrutinib targets not only CLL cells directly, but also myeloid cells which express BTK, as well as T cells via targeting IL-2-inducible T-cell kinase (ITK), a kinase that is very similar to BTK and essential for T-cell effector function. (Long et al., 2017; Stadler et al., 2017; Stiff et al., 2016; Dubovsky et al., 2013) These effects most likely result in altered immune cell functions and might contribute to the treatment effect of Ibrutinib in CLL, but are presumably also involved in the increased risk for infections that are observed under Ibrutinib treatment. Gunderson et al. have recently shown that BTK inhibition by Ibrutinib reprograms TAMs towards stimulatory phenotypes and suppresses tumor growth in pancreatic cancer. (Gunderson et al., 2015) Furthermore, Ibrutinib decreases the production of inflammatory cytokines like TNFα from macrophages. (Chang et al., 2011) On the other hand, BTK inhibition in DCs can abrogate their activation. (Lougaris et al., 2014) Therefore, combination of Ibrutinib and immunotherapeutic approaches targeting myeloid cells offers a novel strategy in CLL therapy that should be thoroughly evaluated.

Indirect effects on cells in the CLL microenvironment were also observed for Idelalisib, another small molecule inhibitor that is approved for CLL treatment and that targets PI3Kδ. (Herman et al., 2010) This PI3K isoform is expressed mainly in lymphocytes, and besides blocking B-cell receptor signaling, it also impairs T-cell effector functions. Myeloid cells do not express PI3Kδ, but PI3Kγ and recent work by Kaneda et al. has demonstrated that PI3Kγ activity in macrophages controls a critical switch between immune stimulation and suppression during inflammation and cancer. (Kaneda et al., 2016) Inhibition of PI3Kγ promoted prolonged NF-κB activity and thereby an immunostimulatory transcriptional program in macrophages that restored $CD8^+$ T-cell activation and cytotoxicity. In mouse models of solid cancers they further demonstrated that PI3Kγ inhibition synergizes with checkpoint inhibitor therapy to promote tumor regression and increased survival of mice.

7.2. Tumor-associated macrophages as therapeutic targets

Due to the prominent pro-tumoral role of M2-like TAMs, the notion of TAM re-education became the focus of multiple immunotherapeutic approaches. Hagemann et al. showed that specific inhibition of NF-κB signaling in TAMs can result in re-educating them to switch to an M1-like or classical inflammatory phenotype, which results in pronounced tumor regression. (Hagemann et al., 2008) Considering the key role of colony-stimulating factor 1 (CSF1) in monocyte survival, chemotaxis and differentiation to macrophages, inhibition of its receptor CSF1R has been intensively used to manipulate TAMs. (Noy and Pollard, 2014) Of interest, CSF1R blockade resulted in reprogramming of TAMs into M1 phenotypes and was accompanied by encouraging responses in mouse models of glioma as well as cervical and mammary tumors. (Pyonteck et al., 2013; Strachan et al., 2013) Combinations of this approach with chemo- or targeted therapy, including checkpoint blockade resulted in improved treatment responses in mouse models of various solid tumors as well as more recently of multiple myeloma. Here, macrophage-induced chemoresistance was shown to be overcome by CSF1R blockade via partial depletion and M1 polarization of macrophages. (Wang et al., 2017)

There is only little evidence so far for successful therapeutic targeting of TAMs in CLL. Galletti et al. showed that blockade of CSF1R signaling in immunocompromised mice that were transplanted with the CLL cell line MEC-1 resulted in reduced leukemic cell load, especially in the BM, and increased circulating leukemic cells. (Galletti et al., 2016) In this model, targeting TAMs with anti-CSF1R and MEC-1 cells with anti-CD20 antibody GA101 provided a survival benefit for the mice. Monotherapy with anti-CSF1R antibodies in the Eμ-TCL1 adoptive transfer model resulted in a considerable reduction of monocyte numbers in the blood and spleen, but this was not associated with a

significant reduction in tumor load (Yazdanparast, Seiffert et al., unpublished). In that same mouse model, depletion of myeloid cells with liposomal clodronate controlled disease development very effectively, (Hanna et al., 2016) implicating that either CSF1R-independent myeloid cells are of pathological relevance, or depletion efficiency obtained with anti-CSF1R is not sufficient to impair CLL outgrowth.

The clinical activity of the immunomodulatory drug lenalidomide in CLL is based on direct effects on the tumor as well as on cells in the microenvironment. (Schulz et al., 2013; Ramsay et al., 2008; Fecteau et al., 2014; Seiffert, 2014) Lenalidomide was shown to alter cytokine secretion and migration of NLCs, as well as the capacity of these cells to support CLL cell survival. (Schulz et al., 2013; Fiorcari et al., 2015)

Gautam et al. recently showed that IFNγ can re-educate NLCs and shift them toward an effector-like state with enhanced Fcγ receptor-mediated cytokine production as well as Rituximab-mediated phagocytosis of CLL cells. (Gautam et al., 2016) These results suggest that therapies promoting local IFNγ production may be effective adjuvants for antibody therapy in CLL.

7.3. Potential targets for novel immunotherapeutic approaches in CLL

Thus far, clinical experience using immune checkpoint blockade in patients with CLL is limited. A clinical trial with Pembrolizumab, a humanized anti-PD-1 antibody, in patients with relapsed CLL or Richter transformation showed no treatment benefit for CLL patients, but objective response in 4 out of 9 patients with Richter transformation which could change the landscape of therapy for this group of patients if further validated. (Ding et al., 2017) Additional studies with anti-PD-1 and anti-PD-L1 antibodies, also in combination with drugs that are clinically used for CLL, are ongoing (NCT03153202) or need to be established to evaluate the potential of these promising new therapeutics for CLL.

As aberrant expression and activity of the immunosuppressive enzyme IDO1 has been discovered in several tumor entities, inhibitors targeting IDO1 have been developed and are currently tested in multiple cancer entities. (Brochez et al., 2017) Preliminary results indicate that combination of IDO1 inhibition with other anti-cancer drugs, e.g. with immune checkpoint blockade seem encouraging, as recently shown for melanoma patients. (Indoximod Combo Triggers Responses in Melanoma, 2017) The relevance of IDO1 as therapeutic target in CLL needs to be explored in the future.

As we observed a CCR2-dependent recruitment of monocytes in the Eμ-TCL1 mouse model, (Hanna et al., 2016) targeting its ligand CCL2 with the monocloncal antibody Carlumab, as currently tested in clinical trials for prostate cancer and other tumors, (Pienta et al., 2013) might be of potential interest in CLL as well. In addition, targeting IL-4, IL-13, or IL-6 or their receptors is a potential strategy to impact on the polarization or function of TAMs which is currently explored in pre-clinical or clinical trials for various solid cancers.

In conclusion, there is a wealth of indications for a pathological role of myeloid cells in CLL and considering the success of therapeutic targeting of these cells in other cancers, we need to explore this potential to develop novel and improved treatment strategies for CLL. Rational combinations of drugs that target the disease from different angles will help us to induce long-lasting treatment success and hopefully also cure for CLL patients.

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